

Chemical, fatty acid, volatile oil composition and antioxidant activity of shade dried neem (*Azadirachta Indica* L.) flower powder

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Article history

Received: 3 July 2012
Received in revised form:
16 February 2014
Accepted: 19 February 2014

Keywords

Azadirachta indica
Antioxidant activity
Chemical composition
Lipids
Neem flowers
Volatile oil

Abstract

Shade dried neem (*Azadirachta indica*) flower powder (NFP) was evaluated for composition, fatty acid profile, volatile oil composition and antioxidant activity. NFP was rich in protein (17.34%), fiber (12.32%) and ash (9.16%). The volatile oil (0.07%) contained 85% of caryophyllene. The lipid yield of NFP was 12%, which was composed of equal quantities (45 ± 1%) of saturated (SFA) and poly unsaturated fatty acids (PUFA). In SFA, the palmitic (31.76%) was major, in PUFA, linoleic (18.57%), linolenic (12.64%) and oleic (9.74%) were higher. Arachidonic (20:4) and docosatrienoic acids (22:3) were also present to an extent of 7.38% and 5.7%, respectively in the total lipid. NFP showed 25.31% radical inhibition with 0.6 mg and 58.50% at 2 mg and comparable iron reducing power. The results reveal that neem flower volatile fraction and lipid can be further explored for pharmaceutical or food applications.

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Introduction

Neem (*Azadirachta indica*) tree belongs to the family of *Meliaceae*, is a large ever green tree growing up to 60 ft high in the dry tropical forests of India, Sri Lanka, Burma, Pakistan, tropical Australia and Africa. Neem twigs are used as tooth brushes in India from times immemorial. Neem flowers are white in color, generally 5 mm long and have a peculiar fragrance and produce nectar. The flowering period is generally between the months of January and April. Buds normally open in the afternoon, emanating a strong smell during night. Flowers are used in pharmaceutical, food and cosmetic industries. During the flowering season, large quantities of flowers fall on earth and go wasted. Neem flowers are used in making a traditional chutney along with mangoes, tamarind, jaggery, salt, chilly and coconut during the Indian New Year Day to mark the six tastes of bitterness, sourness, astringent, sweet, salt and pungent tastes.

The seed kernels constitute 50-60% of the seed weight and 25% of the fruit. The fat content of the kernels ranges from 33-45% (Eckey, 1954). The fruit yield per tree is 37-55 kg. Neem oil (40% yields) is usually opaque, bitter and inedible but can be processed into non bitter edible oil with 42-50% oleic acid and 15% linoleic acid. Rat feeding studies on this refined oil indicated that it was suitable for use as edible oil (Rukmini, 1987). The neem tree provides many useful compounds that are used as pesticides and could be applied to protect stored seeds against

insects (Rahim, 1998; Lale and Abdulrahman, 1999). The beneficial health effects, such as blood sugar lowering properties, anti-parasitic, anti-inflammatory, anti-ulcer and hepatoprotective effects and the toxic effects based on data from human and animal studies and its modifying effects on prevention of gastric carcinogenesis were reviewed (Arivazhagan *et al.*, 2004; Sara, 2004). Chilli was treated with neem seed extract and the growth of aflatoxin *A. flavus* was monitored. The extract, at 5% level and its essential oils inhibited fungal growth (Fandohan *et al.*, 2004; Ajith Kumar and Naik, 2006).

Neem seed has high nutritional potential for livestock and de-bittering of the seed by solvent extraction was reported by Udayashekar (1987). Tignic acid is the principle component responsible for the distinctive odour of neem seed (Iwu, 1993) and sulphur containing compounds like nimbin, nimbidin and nimboesterol were reported to be responsible for the bitter taste (Karkar, 1976). A de-bittered protein powder with protein content of 56% was prepared and studied its protein solubility varying the pH and sodium chloride concentration (Usman *et al.*, 2005).

The effect of extracts of flowers of the neem tree on the levels of cytochrome P450, aniline hydroxylase (ANH) and aminopyrine-N-demethylase (AMD) as well as its capacity to activate the mutagenicity against aflatoxin B₁ (AFB₁) and benzo[a]pyrene (BaP), and to induce glutathione S-transferase (GST) in rat liver were assessed. It was found that feeding of the diets containing 12.5% neem flowers for 2 weeks strongly enhanced GST activity and extracts of neem flowers

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possessed chemo-preventive potential (Kusamran *et al.*, 1998).

Thus, many applications are listed for the neem seeds and its oil as pest repellents, seed treatment in agriculture and as mosquito repellants. Though, raw flowers, flower extract and flower powder find applications in pharma, food and cosmetic products, literature is scanty on the nutritional, fatty acid and essential oil components of the flowers. In the present study, the shade dried neem flower powder was analysed for nutritional composition, fatty acid and essential oil components. The methanol extract of the neem flower powder was evaluated for antioxidant activity by DPPH radical scavenging activity and iron reducing power.

Materials and Methods

Materials

Neem flowers were collected from the trees of CFTRI-Resource Centre Campus, Hyderabad, India. Solvents and chemicals used were of laboratory and analytical grade respectively were purchased from M/s. Loba Chemie, Mumbai, India. Standard fatty acid methyl esters (FAMES, C4-C24) were purchased from M/s. Sigma-Aldrich, St Louis, USA.

Shade drying and preparation of neem flower powder

The collected flowers were separated from foreign material and cleaned from twigs, spread in trays and allowed to dry at (RT) room temperature ($28 \pm 2^\circ\text{C}$) for 48 h. The dried flowers are ground in a laboratory mixer/grinder (Sumeet Food Processor, Nasik, India) and resultant neem flower powder (NFP) is passed through BS 30 mesh (500μ) and packed in metallised polyester-polyethylene pouches. The NFP was stored at RT for further experiments.

Chemical analysis

The chemical components such as ash, protein, crude fat and crude fibre were analyzed by standard methods (AOAC, 1995). Moisture content in NFP was measured by immiscible solvent (toluene) distillation method using Dean and Stark apparatus. The percent carbohydrate content was calculated by difference using the following equation:

$$= 100 - (\text{sum of moisture, total ash, crude fat, crude protein and crude fibre})$$

The energy value was calculated by using following expression and reported as kcal/100 g NFP.

$$= 9 \times \% \text{ fat} + 4 (\% \text{ protein} + \% \text{ carbohydrates})$$

Estimation of phosphorous content

Phosphorous content was measured by taking 5 ml of ash solution (6 N HCl) and reacting with 2 ml of molybdic acid to form the phosphomolybdate, which on treatment with 5 ml of amino naphthol sulphonic acid (ANSA) reagent results in molybdenum blue. The contents volume was made up to 50 ml with water. The contents were allowed at RT for 10 min and the colour was read at 650 nm. Potassium dihydrogen orthophosphate (KH_2PO_4) was used as standard phosphorous reagent (Ranganna, 1986).

Estimation of volatile oil

NFP (100 g) was dispersed in 500 ml distilled water in a round bottom flask and the volatile oil was recovered by hydrosteam distillation using a Clevenger apparatus. The collected volatile oil was measured and reported as ml volatile oil / 100 g NFP.

Extraction of total lipid

NFP (100 g) was extracted with a mixture of solvents (chloroform-methanol, 2:1, v/v) at RT according to the reported method with a minor modification (Folch *et al.*, 1957). The NFP to solvent ratio of 1:3 was maintained and the extraction was carried out using a magnetic stirrer. The procedure was repeated for four times and the pooled solvent extracts were washed with water several times to remove methanol completely. The chloroform layer was dried over anhydrous sodium sulphate and total lipid was recovered by distillation in a rotary vacuum evaporator at $\leq 50^\circ\text{C}$. The obtained total lipid was packed in a glass bottle and stored at RT for further experiments. Phosphorous present in the total lipid was estimated as mentioned in chemical analysis section.

Estimation of free fatty acid

The free fatty acid content (FFA) in total lipid was estimated by dispersing 1 g in previously neutralized ethanol. The contents were heated on a hot plate for complete dispersion of lipid in solution and were titrated with 0.1 N KOH using phenolphthalein as indicator and FFA content was calculated and expressed as percent oleic acid (Ranganna, 1986).

Estimation of peroxide value

The lipid sample (2 g) was dispersed in a solvent mixture of 25 ml (acetic acid: chloroform, 60:40, v/v). The contents were treated with 1 ml potassium iodide solution (4 g KI in 3 ml water) and kept at RT

in dark for 10 min. The contents were mixed with 50 ml distilled water and titrated with 0.1 N sodium thiosulphate. The end point was noted using starch as indicator. The peroxide value was calculated and expressed as meq.O₂/kg lipid (Ranganna, 1986).

Saponification value (SV)

The lipid (2.5 g) was dispersed in 25 ml of alcoholic KOH (0.5N) and refluxed on a hot plate for 3 h. Completion of saponification is indicated by the absence of any oily matter and appearance of clear solution. The flask and condenser were allowed to cool and washed the inside of the condenser with about 10 ml of hot ethyl alcohol neutral to phenolphthalein. The contents were titrated with standard HCl (0.5N). A blank determination was carried out and SV was calculated as follows

$$S.V. = \frac{\text{Blank titre} - \text{sample lipid titre} \times \text{Normality of KOH} \times 56.01}{\text{Weight of lipid (g)}}$$

Unsaponifiable matter

The total lipid (2.5 g) is taken into a round bottom flask and added with 25 ml of alcoholic KOH (0.5N) solution and refluxed for 3 h. The heating was stopped, the condenser was washed with alcohol and the contents were extracted into 25 ml hexane for 3 times and the pooled extracts were washed with sufficient water to remove the alkali. The extracts were dried over anhydrous sodium sulphate and distilled using a rotary vacuum distillation unit. The extract was dried in oven at 100°C for 1 h and weighed. The fatty acid content of the unsaponifiable matter was determined and subtracted from the weight and reported as unsaponifiable matter (Ranganna, 1986).

$$\% \text{ Unsaponifiable matter} = \frac{\text{Weight of unsaponifiable matter} \times 100}{\text{Weight of lipid taken}}$$

Fatty acid and essential oil composition by GC and GC-MS

The fatty acid methyl esters (FAME) of total lipid from NFP were prepared by using mixture of sulphuric acid in methanol (2%, v/v). The FAMES were analysed by Gas chromatography and Gas chromatography - Mass spectrometry as per the method reported (Prabhakara Rao *et al.*, 2010). The GC-FID analyses were performed with an Agilent 6850 series gas chromatograph equipped with an FID detector. A DB-225 capillary column (30 m × 0.25 mm i.d) was used. The column temperature was initially maintained at 160°C for 2 min, increased to 220°C at 6°C/min, and finally maintained for 10 min at 220°C. The carrier gas was nitrogen at a flow rate of 1.5 ml/min. The injector and detector temperatures were maintained at 230 and 250°C, respectively with

a split ratio of 50:1. In case of volatile oil, the GC conditions were altered by modifying the temperature programming as 70°C - 4°C /min. -160°C using the same column as for the lipids.

The GC-MS analyses was performed using an Agilent (Palo Alto, USA) 6890N gas chromatograph equipped with a HP-5 MS capillary column (30 m × 0.25 mm i.d.) connected to an Agilent 5973 mass spectrometer operating in the EI mode (70 eV; m/z 50 – 550; source temperature 230°C and a quadruple temperature 150°C). The column temperature was initially maintained at 200°C for 2 min, increased to 300°C at 4°C/min, and maintained for 20 min at 300°C. The carrier gas was helium at a flow rate of 1.0 mL/min. The inlet temperature was maintained at 300°C with a split ratio of 50:1. Structural assignments were based on interpretation of mass spectrometric fragmentation and confirmed by comparison of retention times as well as fragmentation pattern of authentic compounds and the spectral data obtained from the Wiley and NIST libraries.

DPPH radical scavenging activity

Antioxidant activity of NFP was determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity according to the reported method (Nanjo *et al.*, 1996) with minor modifications. NFP extracts in methanol equivalent to 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 and 2 mg were dispersed in 1 ml of methanol to which 4 ml of methanolic solution of DPPH (0.004%) was added and the contents were incubated at RT for 30 minutes. The optical density of the samples was read at 517 nm. The solution without NFP was taken as control. The DPPH radical scavenging activity of NFP was compared with a synthetic phenolic antioxidant, butylated hydroxytoluene (BHT). The percentage inhibition was calculated using the following expression.

$$I\% = \frac{\text{Absorbance of control} - \text{Absorbance of NFP} \times 100}{\text{Absorbance of control}}$$

Reducing power

The iron reducing power of neem flower powder was determined following a reported method (Yildirim *et al.*, 2001). Aliquots of 0.2 to 1 ml of extracts of NFP in methanol equivalent to 0.2 to 1.0 mg neem flower powder were taken in 20 ml test tubes and the volume was made up to 1 ml with methanol. Phosphate buffer (2.5 ml) and 1% solution of potassium ferricyanide (2.5 ml) were added and the contents of test tubes were incubated for 20 min at 50°C. Trichloroacetic acid (10%, 2.5 ml) was added to each test tube and centrifuged at 1073 x g for 10 min. Supernatant from each test tube (2.5 ml)

was dispersed in 2.5 ml of distilled to which 0.5 ml of 0.1% ferric chloride was added and thoroughly vortexed for 2 min. The optical density of the colour developed was measured at 700 nm. The reducing power of standard synthetic antioxidant, BHT was measured for quantities ranging between 4 and 32 μg for comparison.

Statistical analysis

Nutritional quality, total lipid extraction, lipid analysis and estimation of volatile oil were carried out in three replications and the mean values with standard deviation (mean \pm SD) computed using MS Excel, 2007 were reported. The GC, GC-MS analyses of fatty acid methyl esters and volatile oil were performed in duplicate and average values were presented.

Results and Discussion

The photographs of inflorescence of neem (*Azadirachta indica*) and shade dried neem flowers are presented in Figure 1.

Chemical composition

It was observed that NFP possessed higher amounts of protein, fiber and ash, 17.34, 12.32 and 9.16% respectively (Table 1). The crude fat of NFP was accounted for 5.16% by soxhlet extraction. The phosphorous was found to be an extent of 135 mg/100 g NFP.

Quality of volatile oil

Neem flower powder yielded volatile oil to an extent of 0.08% on dry weight basis. The GC and GC-MS results showed that the volatile oil is rich in sesquiterpenes caryophyllene (56.03%), caryophyllene oxide (17.41%) and α -Caryophyllene (12.10%). α -Caryophyllene (monocyclic sesquiterpene) and caryophyllene (bicyclic sesquiterpene) are isomers. α -Caryophyllene (humulene) was reported to be effective against inflammatory diseases, which possessed inhibitory effects on tumor necrosis factors in rats (Fernandes *et al.*, 2007). Bicyclo (5, 2, 0) non-1-ene (3.44%), cyclohexane (3.65%) and copaene (1.55%) were also present (Table 2). Studies on *Murraya koenigii* flowers reported a yield 0.21% of volatile oil on dry weight basis and mono and sesquiterpenes constituted the major components (87%). Cis-ocimene (34.1%), α -pinene (19.1%), γ -terpinene (6.7%) and β -caryophyllene (9.5%) were observed to be predominant in *Murraya koenigii* flowers (Walde *et al.*, 2006).

Table 1. Chemical composition and total lipid analysis of NFP^a

| Parameter, % | Value |
|--|------------------|
| Moisture | 9.31 \pm 0.33 |
| Total ash | 9.16 \pm 0.25 |
| Crude protein | 17.34 \pm 0.43 |
| Crude fat | 5.16 \pm 0.47 |
| Crude fibre | 12.32 \pm 0.63 |
| Carbohydrates by difference | 46.71 \pm 1.28 |
| Energy (kcal/100g) | 303 \pm 2.34 |
| Phosphorous (mg/100g) | 135 \pm 0.84 |
| Volatile oil | 0.07 \pm 0.01 |
| Total lipid analysis | |
| Yield, % | 11.98 \pm 0.50 |
| Phosphorous (mg/100 g) | 119 \pm 0.64 |
| Free fatty acids (as % oleic acid) | 3.06 \pm 1.14 |
| Peroxide vale (mill. eq. oxygen/kg of fat) | 26.6 \pm 1.36 |
| Saponification value (mg KOH per g lipid) | 89.9 \pm 1.67 |
| Unsaponifiable matter (%) | 1.93 \pm 0.03 |

^aValues are mean of triplicate analyses with \pm SD

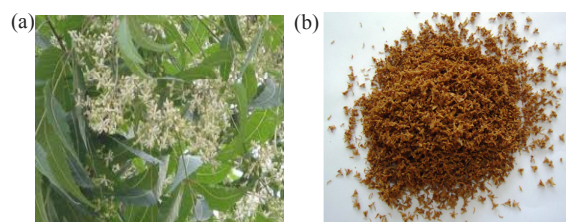


Figure 1. Neem (*Azadirachta indica*) (a) Inflorescence; (b) Shade dried neem flowers

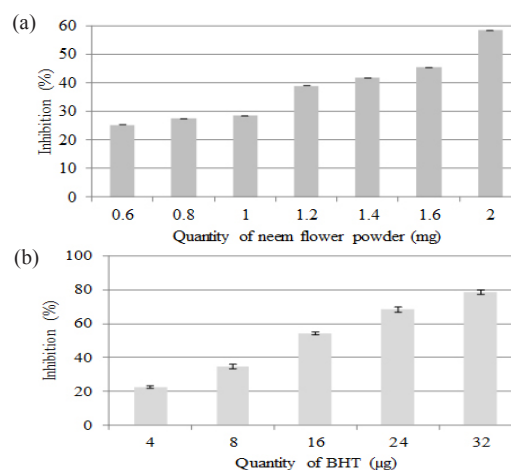


Figure 2. DPPH radical scavenging activity (a) Neem flower powder (b) BHT; Vertical error bars represent standard deviation

Quality of total lipid

The total lipid yield of NFP by extraction using chloroform/methanol (2:1, v/v) was 11.98%. Higher free fatty acid (3.06%) and peroxide value (26.6 meq. O_2/kg) were noticed. The saponification value was 89.9 mg of KOH per g of lipid. Unsaponifiable matter was found to be 1.93%. Phosphorous was found to be 119 mg/100 g lipid (Table 1). In contrast, the total lipid of *Sterculia urens* seed was 39.2%. The free fatty acid content and peroxide value of the freshly extracted *Sterculia urens* lipid was found to be 2.6% and 1.30 meq. of oxygen/kg respectively (Narsing Rao *et al.*, 2012). In jatropha seed oil, a FFA content of 2.23% and POV of 1.93 meq./kg was reported (Akbar *et al.*, 2009). The quantity of free fatty acids

Table 2. Volatile oil composition of NFP^a

| Compound | RT (min) | Peak area % |
|------------------------------|----------|-------------|
| Copaene | 13.21 | 1.55 |
| Bicyclo [5, 2, 0] non -1-ene | 13.58 | 3.44 |
| Caryophyllene | 13.75 | 56.03 |
| α -Caryophyllene | 14.10 | 12.10 |
| Cyclohexene | 14.63 | 1.50 |
| Caryophyllene oxide | 15.56 | 17.41 |
| 2H - Indene 2-one | 15.84 | 1.54 |
| Cyclohexane | 17.25 | 3.65 |
| 1,2 Benzenedicarboxylic acid | 18.21 | 2.78 |

^aValues are mean of duplicate analysesTable 3. Fatty acid composition of total lipid in NFP^a

| Fatty acid | % composition |
|-----------------|---------------|
| 8:0 | 0.34 |
| 10:0 | 0.11 |
| 11:0 | 0.34 |
| 12:0 | 1.00 |
| 13:0 | 0.45 |
| 14:0 | 1.90 |
| 15:0 | 0.78 |
| 16:0 | 31.76 |
| 17:0 | 0.34 |
| 18:0 | 2.90 |
| 20:0 | 1.34 |
| 22:0 | 2.13 |
| 24:0 | 1.68 |
| Saturated | 45.07 |
| 18:1 | 9.74 |
| 20:1 | 0.45 |
| Monounsaturated | 10.19 |
| 18:2 | 18.57 |
| 22:2 | 5.70 |
| 18:3 | 12.64 |
| 22:3 | 0.45 |
| 20:4 | 7.38 |
| Polyunsaturated | 44.74 |

^aValues are mean of duplicate analyses

was reported to affect the formation of soap or biodiesel manufacture and a free fatty acid content < 1% was recommended (Goodrum, 2002). The higher FFA, POV, unsaponifiable matter of the lipid and its bitter taste indicated that physical refining may be recommended if its food uses are to be explored.

Fatty acid composition of total lipid

The results of GC and GC-MS analysis of fatty acid composition for NFP total lipid are presented in Table 3. The SFA and PUFA each was contributed an amount of ~45% and mono unsaturated accounted 10% of total lipid. The fatty acid composition of NFP was characterized by substantial amounts of palmitic (31.76%), linoleic (18.57%), linolenic (12.64%), oleic (9.74%) and stearic acids (2.90%). The total lipid was composed of high concentration of unsaturated fatty acids, which accounts 54%. The polyunsaturated fatty acid content of 36.7% in the total lipid of *Sterculia urens* cotyledons and ~38% in sesame seed was reported (Kanu, 2011; Narsing Rao et al., 2012). Palmitic acid (14.2%), oleic acid (44.7%) and linoleic acid (32.8%) are constituents of

oil extracted by hexane from jatropha seeds (Akbar et al., 2009). Palm oil containing high saturated fats (~50%) was reported to be suitable for food applications and pharmaceutical industries (Che Man et al., 1999; Dai et al., 2002; Soh et al., 2006; Matthaus, 2007).

Higher saturated (45.07%) and polyunsaturated (44.75%) and lower monounsaturated (10.19%) fatty acids were noticed in neem flower powder lipid. The ratio of polyunsaturated to saturated fatty acids (PUFA/SFA) was found to be 0.99; the ratio of polyunsaturated to monounsaturated fatty acids (PUFA/MUFA) was 4.39 in total lipid. The ratio of polyunsaturated to saturated fatty acids was found to be lower (0.99) and the ratio of polyunsaturated to monounsaturated fatty acids was very high (4.39). PUFA + MUFA/SFA ratio was found to be 1.22. Apart from these fatty acids considerable amounts of unconventional polyunsaturated fatty acids such as 20:4 (7.38%), 22:2 (5.7%) and very lower quantities of 22:3 (0.45%) were also observed in the total lipid. The presence of arachidonic acid (20:4) is a rare phenomenon in plant lipids, which is commonly observed in animal fats. In jojoba seeds, it was observed that an unconventional monounsaturated fatty acid, 20:1 was found to be a major fatty acid (66.3%) (Maria et al., 2009). The ratio of PUFA/SFA is generally used to evaluate the nutritional value of lipid. The effects PUFA + MUFA/SFA on plasma and liver lipid concentrations in rats were reported (Chang and Huang, 1998). They concluded that the prerequisites for keeping low plasma and liver lipid concentration are low MUFA/SFA ratio and high PUFA/MUFA ratio, and PUFA + MUFA/SFA ratio not to exceed 2, which is in good agreement with neem flower lipids in the present work (1.22).

Antioxidant activity

Greater DPPH radical scavenging activity was found in NFP, which increased with increase in quantity. NFP extract equivalent to 0.6 mg showed an inhibition of 25.31% and it was 58.50% with 2 mg. Under similar experimental conditions, butylated hydroxytolulene (BHT) showed 22.61% inhibition for 4 μ g and 78.53% for 32 μ g (Figure 2).

Reducing power of neem flower powder in ferric chloride showed an optical density of 0.086 for 0.2 mg and 0.489 for 1 mg, whereas the optical density readings for synthetic antioxidant, BHT were 0.031 for 4 μ g and 0.126 for 32 μ g (Figure 3). The exhibition of high degree of antioxidant activity of NFP may be due to the high amounts of sesquiterpenes such as caryophyllene (56%) or due to phytosterols present in the methanolic extracts. The

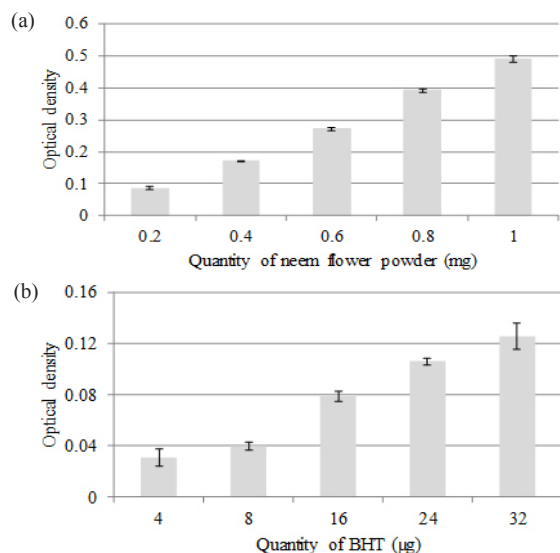


Figure 3. Ferric reducing power (a) neem flower powder (b) BHT; Vertical error bars represent standard deviation

essential oil of the aerial parts of *Teucrium orientale* was reported to be constituted of linalool (28.6%), caryophyllene oxide (15.6%), 1,8-cineol (4.5%), β -pinene (8.7%), 3-octanol (9.5%), β -caryophyllene (7.3%) and germacrene-D (4.1%). Among the volatile oil and methanolic extracts, the greatest inhibition capacity of 95.21% was found for the water soluble polar fraction of the methanol extract (Amiri, 2010). Methanolic extracts of wild edible mushroom species from whole mushroom, cap and the stipe, separately were screened for their reducing power and free radical scavenging capacity and it was observed that the cap showed maximum activity (Isabel *et al.*, 2007).

Conclusion

In conclusion, the large quantities of neem flowers are wasted during flowering season every year. The neem tree has several applications in medicine, agriculture and cosmetics. The results revealed that the neem flower powder could be a good source of lipid with polyunsaturated fatty acids such as oleic, linoleic, linolenic and few unconventional fatty acids. The volatile oil has characteristic flavour from caryophyllene. The flower powder exhibited higher DPPH radical scavenging activity and iron reducing power. The results are encouraging for proposing in vivo animal studies for determining the physiological activities of neem flower extracts for their application in food and pharmaceutical industries.

Acknowledgement

The authors thank Director, CFTRI, Mysore for his interest in the work and permission to publish the

data.

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